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The yeast permease Acr3p is a dual arsenite and antimonite plasma membrane transporter

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ABSTRACT

The Acr3p permease from the yeast *Saccharomyces cerevisiae* is a prototype member of the arsenical resistance-3 (Acr3) family of transporters, which are found in all domains of life. Remarkably little is known about substrate specificity, localization and regulation of Acr3 proteins. Here, we show that the yeast Acr3p mediates not only high-level resistance to arsenite but also moderate tolerance to antimonite. The *acr3* deletion mutant shows increased sensitivity to antimonite. In addition, overexpression of the *ACR3* gene complements antimonite sensitivity of cells lacking the vacuolar ABC transporter Ycf1p. Moreover, both antimonite and arsenite induce transcription of the *ACR3* gene resulting in the accumulation of Acr3 transporter at the plasma membrane. However, antimonite is much weaker inducer of the *ACR3* gene transcription comparing to arsenite. Interestingly, the presence of metalloids does not influence either stability of Acr3 protein or its intracellular localization suggesting that Acr3p is mainly regulated at the transcriptional level. Finally, transport experiments confirmed that Acr3p indeed mediates efflux of antimonite and thus possesses a dual arsenite and antimonite specificity.

1. Introduction

Highly toxic arsenic is abundantly present in the environment and affects all organisms, including humans. Arsenic is classified as a human carcinogen [1] but together with related metalloid antimony is also used as a drug in anticancer [2] and antiprotozoan therapies [3]. Several strategies have been developed by organisms to cope with toxic metalloids, including inhibition of metalloid accumulation into the cell, chelation by metal-binding proteins and sequestration into intracellular compartments [4,5]. However, the most effective mode of metalloid detoxification is extrusion out of the cell. Up to date, four different types of transporters have been shown to confer efflux of arsenite (As(III)) and antimonite (Sb(III)): the ATP-binding cassette (ABC) transporters of the multidrug resistance-associated protein (MRP) family, the aquaglyceroporins and two unrelated but metalloid-specific families of transporters, ArsB and Acr3 [4,5].

Mammalian Mrp1 and Mrp2 pump As(III) conjugated to glutathione out of the cell into bile and blood [6]. In addition, members of the MRP family, Ycf1p in *Saccharomyces cerevisiae* [7], Hmt1 in *Schizosaccharomyces pombe* [8], and PgpA in *Leishmania tarentolae* [9], sequester As(III) and Sb(III) in complex with peptides into intracellular compartments. The aquaglyceroporins constitutes the main entrance pathway for $As(OH)_3$ and $Sb(OH)_3$ and contribute to metalloid sensitivity in most organisms [5]. However, the aquaglyceroporins may also facilitate metalloid export: AqpS in the bacterium *Sinorhizobium meliloti* [10] and Fps1p in *S. cerevisiae* [11] are involved in tolerance to arsenate (As(V)) by extruding $As(OH)_3$ formed as a result of As(V) reduction. Moreover, several plant aquaglyceroporins of nodulin26-like intrinsic protein subfamily are also bidirectional metalloid channels [12], while the aquaglyceroporin-9 facilitates extrusion of pentavalent methylated arsenic species and is required for arsenic clearance in mammals [13,14].

The best studied metalloid transporter is the *Escherichia coli* ArsB protein, which is an antiporter exchanging $As(OH)_3$ or $Sb(OH)_3$ for H^+ [15]. However, when the ATPase protein ArsA is expressed, the ArsAB complex is formed which acts as an ATP-driven pump conferring a maximal level of As(III) and Sb(III) resistance [16]. Members of the ArsB family have a 12-transmembrane topology [17] and are similar to the major facilitator superfamily of transporters [18]. Orthologues of the *arsB* gene are often found in the genomes of bacteria and archaea but are absent in eukaryotes [19,20]. In contrast, members of the second family of metalloid transporters Acr3 are found in all domains of life but are particularly widespread in bacteria, archaea and fungi [11,19,20]. The Acr3 family is a distinct group of transporters of 10-transmembrane-spanner topology [19,21], similar to the bile acid:Na+ symporter (BASS) and riboflavin transporter (RFT) families, which together constitute the bile/arsenite/riboflavin transporter (BART) superfamily [22]. In contrast to ArsB, Acr3 transporters seem to display different substrate specificities: Acr3 proteins in *Bacillus subtilis* [23], *Corynebacterium*

Abbreviations: As(III), Sodium arsenite; Sb(III), Potassium antimonyl tartrate; GFP, Green fluorescent protein; MIC, Minimal inhibitory concentration

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glutamicum [19,24], *Alkaliphilus metalliredigens* [19], *S. cerevisiae* [7,25,26] and *S. douglasii* [27] have been reported to confer As(III) resistance, Acr3 from *Synechocystis* sp. mediates tolerance to both As(III) and Sb(III) [28], while the *Shewanella oneidensis* Acr3 is involved in As (V) resistance [29].

The yeast Acr3p is a founder member of the Acr3 family and was the first Acr3 protein shown to mediate efflux of As(III) and confer resistance to arsenicals [7,25,26]. However, little is known about substrate specificity and localization of Acr3p, mechanism of metalloid transport via Acr3p and its regulation. Here, we show that the *S. cerevisiae* Acr3p is localized to the plasma membrane, mediates efflux of not only As(III) but also Sb(III) and is regulated mainly at the transcriptional level.

2. Materials and methods

2.1. Yeast strains, plasmids and growth conditions

Yeast strains and plasmids used in this study are listed in Table 1. Yeast transformations were done by the lithium acetate procedure [30]. Yeast strains were grown in standard rich (YPD) medium or in selective synthetic minimal (SD) medium at 28 °C [31]. Growth assays in the presence of sodium arsenite (As(III)) and potassium antimonyl tartrate (Sb(III)) were carried out as previously described [32].

2.2. RNA extraction, reverse transcription and quantitative PCR

Total RNA was isolated from yeast cells using RNeasy® Mini Kit (Qiagen). Subsequently 3 μg of RNA were treated with DNaseI (RNase-Free, Fermentas) following the manufacturer's instruction. The absence of genomic DNA was verified by PCR using specific primers for the *ACR3* gene: ACR3RT-F (5'-CGGCATACCACTGGGAATT-3') and ACR3RT-R (5'-TGCTTTGTCCCATTGGTGC-3'). Reverse transcription was performed with 1.5 μg of purified RNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instruction. The resulted cDNA was verified by PCR using the primers for the *ACR3* gene. Real-time amplification was performed in 7500 Real-Time (Applied Biosystems) with the Real-Time 2x PCR Master Mix SYBR® kit (A&A Biotechnology) according to the manufacturer's instructions using 1 μl of cDNA and the primers: ACR3RT-F and ACR3RT-R in a total volume of 20 μl. Other conditions and quality assessment of PCR reactions as well as analysis of quantitative PCR results were done as described elsewhere [11].

2.3. Protein extracts and immunoblotting

Total protein was extracted by the trichloroacetic acid method as described previously [33]. Alternatively to get a membrane-enriched protein fraction, cells were broken with glass beads in a lysis buffer (20 mM Tris–HCl, 2 mM $MgCl_2$, 250 mM sorbitol, pH 7.5) supplemented with protease inhibitors and centrifugated at 950*g* for 5 min. Next, the supernatant was centrifuged at 15000 g for 40 min to give a membrane-enriched pellet which was resuspended in a MS buffer (2 mM $MgCl_2$, 10 mM imidazole). Protein extracts were resolved on 10% SDS-PAGE, blotted onto nitrocellulose filters (Bio-Rad) and probed with the anti-GFP antibody (Roche) to detect Acr3p tagged with a green fluorescent protein (GFP), the anti-PSTAIRE antibody (Santa Cruz Biotechnology) to examine the level of cyclin-dependent kinase Cdc28p as a protein loading control or the anti-PMA1 antibody against the plasma membrane H+-ATPase (kindly provided by M. Ghislain, University of Louvain, Belgium) to verify quality of the membrane-enriched protein fraction.

Table 1
Yeast strains and plasmids used in this work.

Strain/plasmid	Genotype/plasmid description	Source/reference
Strains		
W303-1A	*MAT***a** *ura3 leu2 trp1 his3 ade2 can1*	S. Hohmann
RW104	W303-1A *acr3Δ::kanMX*	[32]
RW105	W303-1A *acr3Δ::kanMX ycf1Δ::loxP*	[32]
RW118	W303-1A *ycf1Δ::loxP*	[32]
Plasmids		
YEplac195	2 μ *S. cerevisiae-E. coli* shuttle vector, Amp^R, *URA3*	M. Tamás
YEpACR3	YEplac195 containing the *ACR3* gene	This study
pUG35	CEN *S. cerevisiae-E. coli* shuttle vector, containing the yEGFP3 for C-terminal fusion, Amp^R, *URA3*	[43]
pACR3-GFP	pUG35 containing the *ACR3-GFP* fusion gene under the control of the native *ACR3* promoter	This study

2.4. Fluorescence microscopy

Distribution of GFP-tagged Acr3p in living yeast cells exposed to 0.1 mM As(III) or 1 mM Sb(III) was examined with an Axio Imager M1 upright wide-field fluorescence microscope (Carl Zeiss, Germany) equipped with an illuminator (Zeiss HBO 100), a 100× oil immersion objective (Zeiss Plan-Neofluar 100×/1.30), and a GFP filter set. Images were collected using a Zeiss AxioCam MRc digital color camera and processed with Zeiss AxioVision 4.5 software.

2.5. Transport assays

Sb(III) transport experiments were performed essentially as described previously for As(III) transport [34]. Briefly, exponentially growing cells were exposed to 5 mM Sb(III) and collected at 20-min intervals to measure Sb(III) uptake or treated with 5 mM Sb(III) for 120 min, washed to remove Sb(III) from the medium, and resuspended in fresh medium to determine Sb(III) efflux. Collected culture samples from both uptake and efflux experiments were washed in ice-cold water and centrifuged. The cell pellet was resuspended in water, boiled for 10 min, and centrifuged to collect the supernatant.

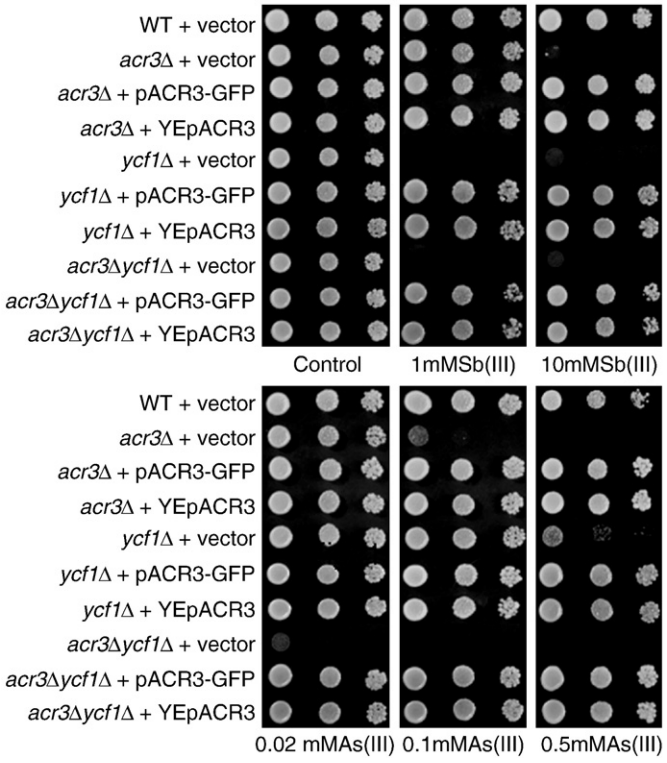

Fig. 1. Metalloid sensitivity phenotype of mutants lacking *ACR3* and *YCF1* genes. Cultures of the indicated yeast transformants were spotted on selective SD plates in the presence or absence of metalloids. Plates were photographed after 3 days at 28 °C.

Table 2
Metalloid resistance phenotype resulting from the overexpression of the *ACR3* gene.

Yeast transformant[a]	MIC[b] (mM)	
	As(III)	Sb(III)
Wild type + YEplac195	1.20	15.0
Wild type + YEpACR3	5.00	20.0
*acr3*Δ + YEplac195	0.10	10.0
*acr3*Δ + YEpACR3	5.00	20.0
*ycf1*Δ + YEplac195	0.70	0.3
*ycf1*Δ + YEpACR3	5.00	15.0
*acr3*Δ *ycf1*Δ + YEplac195	0.02	0.2
*acr3*Δ *ycf1*Δ + YEpACR3	3.00	15.0

[a] Yeast cells were transformed with the multi-copy YEpACR3 plasmid bearing the *ACR3* gene or the empty YEplac195 vector for a control.
[b] MIC is the minimal inhibitory concentration of tested metalloid at which no growth was observed. MIC values were determined on the basis of three independent experiments with identical results using selective synthetic minimal solid medium at 28 °C and 3 days of incubation.

The antimony content of each sample was determined using a flame atomic absorption spectrometer (3300, Perkin Elmer).

3. Results

3.1. Acr3p confers high-level tolerance to As(III) and moderate resistance to Sb(III)

Despite the chemical similarity of As(III) and Sb(III), the *S. cerevisiae* and *B. subtilis* Acr3 proteins have been originally shown to contribute to As(III) tolerance [7,23,25,26] and As(III) efflux [7,25,26] but no increased sensitivity to Sb(III) has been reported leading to the conclusion that Acr3 is a specific As(III) permease. However, in the case of *S. cerevisiae* Acr3p either only low range of Sb(III) concentrations has been tested [7] or probably inactive Sb(III) compound has been used [25,26] suggesting that phenotype of increased Sb(III) sensitivity in *acr3*Δ could be missed. In one of our recent studies on the mechanisms of metalloid resistance in yeast, however, we found that yeast cells lacking the *ACR3* gene or its transcription activator gene, *YAP8*, show increased sensitivity to Sb(III) [35]. In addition, the *ACR3* promoter has been shown to be Sb(III)-responsive [35]. These observations suggested that Acr3p may also contribute to Sb(III) resistance and prompted us to reexamine the role of Acr3p in Sb(III) transport and tolerance.

First, we determined the minimal inhibitory concentration (MIC) of As(III) and Sb(III) for wild type strain and mutants lacking two known metalloid transporters, Acr3p and Ycf1p (Fig. 1, Table 2). Based on the fact that overexpression of *ACR3* highly increases resistance to As(III) [25,26], we also transformed all tested yeast strains with the low-copy and multi-copy plasmids bearing the *ACR3* gene and examined the growth of resulting transformants in the presence of metalloids. In agreement with our previous findings, the *acr3* deletion mutant (*acr3*Δ) was only 1.5-fold more sensitive to Sb(III) (MIC of 10 mM vs. 15 mM for wild type). In contrast, the *ycf1*Δ cells lacking the vacuolar ABC transporter were 50-fold more Sb(III) sensitive (MIC of 0.3 mM). Moreover, the double *acr3*Δ *ycf1*Δ mutant was only slightly less tolerant to Sb(III) than the single *ycf1*Δ mutant. On the other hand, *acr3*Δ showed high sensitivity to As(III) (MIC of 0.1 mM vs. 1.2 mM for wild type), whereas *ycf1*Δ was only slightly As(III) sensitive (MIC of 0.7 mM). These results confirmed the model in which Acr3p plays a major role in conferring As(III) resistance, while Ycf1p is required mainly for Sb(III) detoxification. However, we found that additional copies of the *ACR3* gene fully complemented sensitivity to Sb(III) not only in the *acr3*Δ but also in the *ycf1*Δ background and slightly increased Sb(III) resistance in wild type cells (Fig. 1, Table 2). These findings suggest that Acr3p may contribute to Sb(III) resistance by removing this metalloid from the cytoplasm out of the yeast cell.

3.2. Metalloid-induced expression of Acr3p is regulated at the level of transcription

To confirm a physiological role of Acr3p in mediating tolerance to Sb (III), we followed the mRNA and protein levels for the Acr3 permease fused to GFP as well as its cellular localization in the presence of As(III) and Sb(III). Addition of 0.1 mM As(III) to the medium resulted in a 30-fold induction of mRNA level of the *ACR3* gene (Fig. 2). In the case of Sb (III), 1 mM concentration of this metalloid had to be used to detect a 6-fold induction of *ACR3* mRNA level. In both conditions tested, increasing level of *ACR3* mRNA was accompanied by accumulation of Acr3 protein that was localized to the plasma membrane as determined by Western blot and fluorescence microscopy analysis (Fig. 3). It is important to note that this is the first demonstration of the plasma membrane localization of Acr3p in *S. cerevisiae* cells.

3.3. The plasma membrane localization and protein stability of Acr3p is not regulated by the presence of its substrate

Expression of both ArsB in bacteria and Acr3p in yeast is tightly regulated by the presence of metalloids at the transcriptional level [35–37]. However, metal transporters in yeast are also regulated post-translationally to modulate their stability and trafficking [5,38,39]. On the other hand, the Yap8 transcription factor is stabilized by As(III) to enable activation of the *ACR3* gene transcription [40]. To determine whether As(III) effects stability or intracellular localization of Acr3p, we induced expression of Acr3p-GFP by exposing cells to As(III) for 2 h, washed out As(III) and released the cells in fresh medium to monitor protein level and localization of Acr3p-GFP in the absence of As(III). We found that level of Acr3p slowly decreased after removal of As(III) from the medium (Fig. 4), which is in agreement with the observations obtained by Di and Tamás [40] showing persistence of Yap8p for at least 2 h after washing out As(III). When the cells were

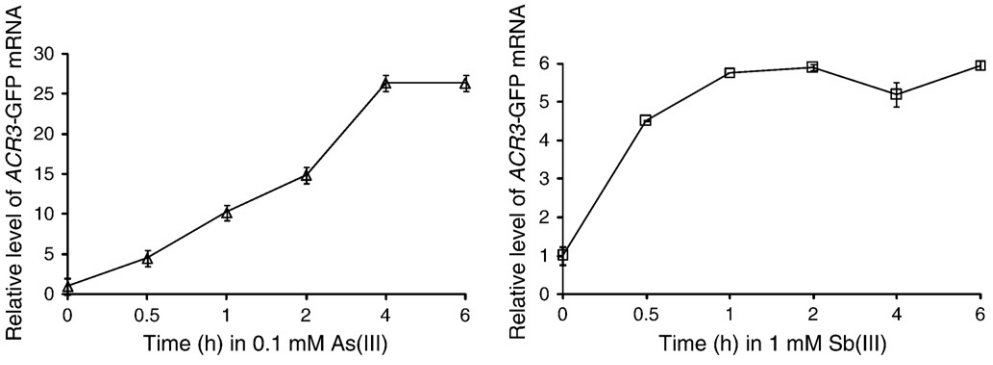

Fig. 2. Transcription of the *ACR3* gene in response to metalloid treatment. Total RNA was isolated from exponentially growing *acr3*Δ cells expressing the *ACR3-GFP* fusion gene (in selective SD medium) that were exposed to 0.1 mM As(III) or 1 mM Sb(III) and analyzed by quantitative PCR.

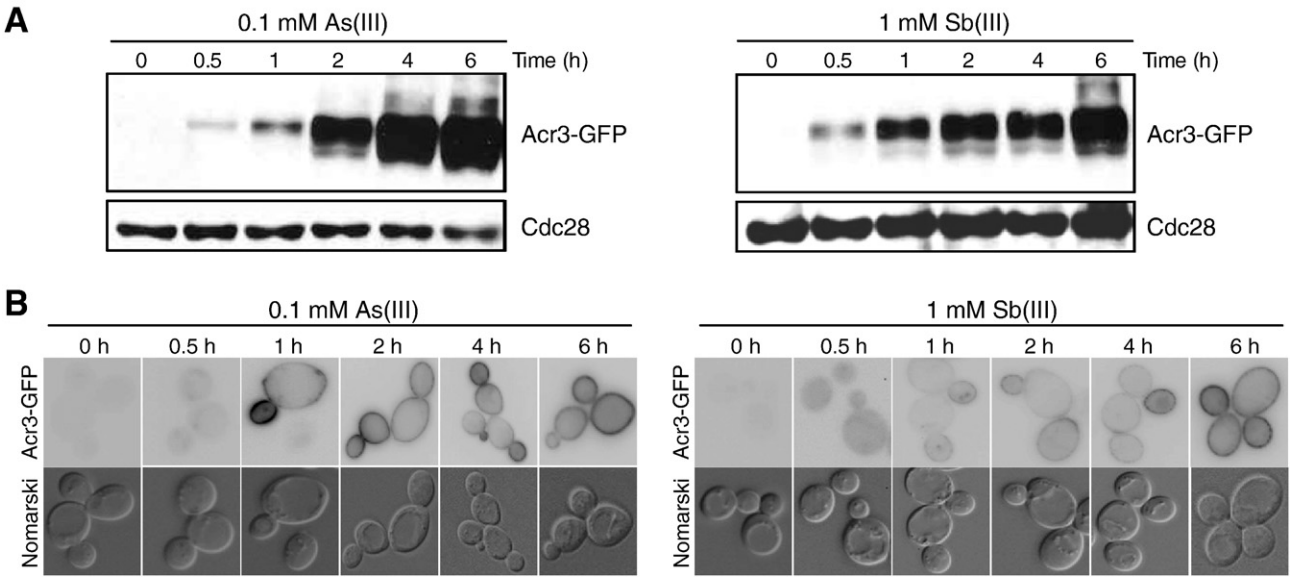

Fig. 3. Expression and localization of Acr3p during As(III) and Sb(III) stress. (A) GFP-tagged Acr3p was expressed from the pUG35 plasmid (pACR3-GFP) in the *acr3*Δ mutant and total protein extracts were prepared for Western blot analysis at the indicated time-points after addition of As(III) or Sb(III) to the media. Blots were probed with the anti-GFP antibody or the anti-PlSTAIRE antibody for detection of Cdc28p as a loading control. (B) Cell samples taken at the same time-points as above were also examined by fluorescence microscopy to determine cellular localization of Acr3p-GFP.

released in a As(III)-free medium containing cycloheximide to inhibit *de novo* protein biosynthesis, Acr3p-GFP level rapidly diminished. Interestingly, addition of As(III) back to the cycloheximide medium did not prevent degradation of remaining Acr3p (Fig. 4). Thus, Acr3p is principally regulated at the transcriptional level, while stability of the Acr3 protein and its plasma membrane localization are not affected by the presence or absence of As(III).

3.4. The Acr3 permease mediates Sb(III) efflux

To confirm the ability of Acr3p to facilitate Sb(III) extrusion, we performed Sb(III) transport studies in wild type and *acr3*Δ cells transformed with either an empty plasmid for a control or the plasmids bearing the *ACR3* gene. It has been already shown that expression of Acr3p leads to decreased uptake of As(III) as a result of As(III) efflux, while the *acr3*Δ mutant shows high content of As(III) and no efflux of As(III) [7,11,26,34]. Thus, we first assayed uptake of Sb(III) into yeast cells. Wild type cells displayed intermediate rate of As(III) influx, while the *acr3*Δ mutant accumulated the highest level of Sb(III). In contrast, expression of the *ACR3* gene from the plasmids significantly reduced uptake of Sb(III) (Fig. 5A). We next loaded yeast cells with Sb(III) and then released in fresh media in the absence of metalloid to determine Sb(III) efflux. Cells expressing Acr3p rapidly reduced the intracelullar concentration of Sb(III), while the *acr3*Δ mutant showed little Sb(III)

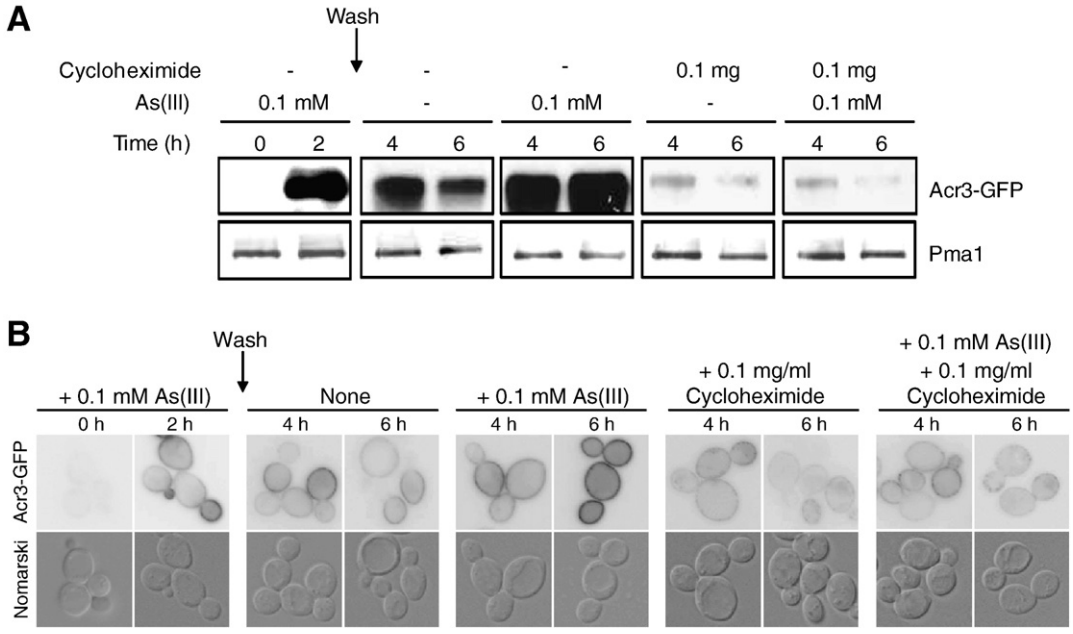

Fig. 4. Stability of Acr3p-GFP in the presence of As(III). First, expression of Acr3p-GFP from the pUG35 plasmid was induced by 2 h-exposure to 0.1 mM As(III). Next, the cell culture was washed to remove As(III) from the medium and resuspended in a fresh medium. Then, the culture was divided in four parts that were left untreated or exposed to 0.1 mM As (III), 0.1 mg/ml cycloheximide, 0.1 mM As(III) and 0.1 mg/ml cycloheximide, respectively. Cell samples were taken at the indicated time-points for preparation of membrane-enriched protein fractions followed by Western blotting analysis (A) and fluorescence microscopy observations (B). Blots were probed for Pma1p which served both as a protein loading control and a quality control of membrane-enriched fractions.

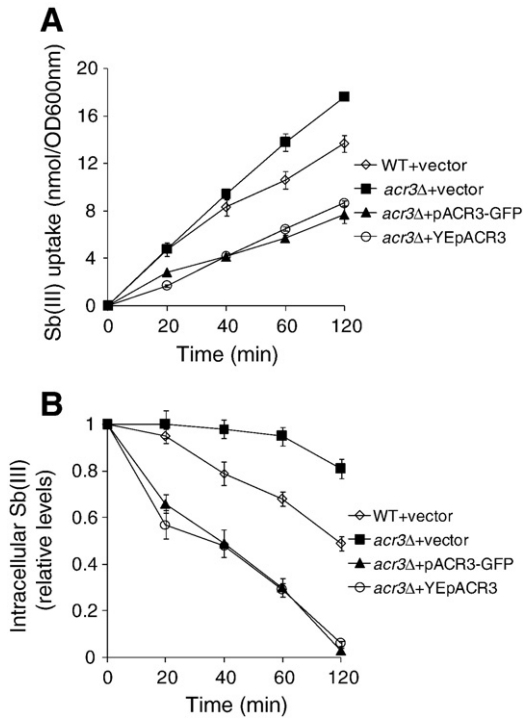

Fig. 5. Sb(III) transport in *S. cerevisiae* cells. (A) Sb(III) accumulation. (B) Sb(III) efflux. Sb(III) transport was assayed in the wild type strain and the *acr3*Δ mutant transformed with the empty YEplac195 plasmid (control), the low-copy pACR3-GFP or the multi-copy YEpACR3 plasmids bearing the *ACR3* gene as described in Materials and methods. Error bars represent the SD of three independent experiments.

export (Fig. 5B). Based on these results, we conclude that Acr3p is capable of transporting Sb(III) out of yeast cells.

4. Discussion

The Acr3p permease constitutes a major detoxification pathway for arsenicals in budding yeast. Overexpression of the *ACR3* gene increases 10-fold resistance to As(III), while yeast cells lacking Acr3p are highly sensitive to both As(III) and As(V) [7,25,26]. In addition, Acr3p-mediated resistance to As(V) requires the presence of arsenate reductase Acr2p which converts As(V) to As(III) [25,41]. Interestingly, both *ACR2* and *ACR3* genes are controlled from a common promoter region which is induced by the transcription factor Yap8p in the presence of metalloids [35,36]. Acr3p has been shown to reduce uptake of As(III) leading to the conclusion that Acr3p is a plasma membrane arsenite permease [7,26]. In contrast to the bacterial ArsB transporter conferring high resistance to As(III) and Sb(III), the *acr3*Δ mutant did not show increased sensitivity to Sb(III) suggesting that Acr3p is not involved in Sb(III) efflux. In support of this notion, Acr3 from *B. subtilis* [23], *C. glutamicum* [19,24], *A. metalliredigens* [19], *S. oneidensis* [29], and several isolates of arsenite-resistant bacteria from soil [20] do not confer cross-resistance to Sb(III). However, it is important to say that up to now transport capacity of Acr3 for Sb(III) has never been studied.

Although the mode of metalloid transport catalyzed by Acr3 protein remains to be established, it has been recently hypothesized that Acr3 may facilitate transport of the arsenic anion $As(OH)_2O^-$ coupled to the membrane potential [19], in contrast to ArsB which transports neutral $As(OH)_3$ in exchange for a proton. This would explain inability of Acr3 proteins to facilitate transport of inorganic antimony which exists probably as $Sb(OH)_3$ at pH 7.2–7.5 in the cytosol of most cells and thus cannot be recognized by anion-specific Acr3. On the other hand, the *Synechocystis* mutant lacking Acr3 orthologue is sensitive to both As(III) and Sb(III) and its expression is highly induced by these metalloids suggesting that some members of Acr3 family may mediate Sb(III) transport and resistance [28].

Having known that the *ACR3* promoter fusion with the *lacZ* gene reporter is moderately activated by Sb(III) [35], we hypothesized that the yeast Acr3p may contribute to Sb(III) tolerance. In this study, we found that yeast cells lacking Acr3p were slightly more sensitive to Sb(III) (Fig. 1, Table 2), showed higher rate of Sb(III) uptake than wild type and little Sb(III) efflux (Fig. 5). These observations strongly suggest that Acr3p is a dual As(III)/Sb(III) permease. However, the question remains why Acr3p contributes more to As(III) tolerance than to Sb(III)? Similarly to bacterial cells, in the yeast cytosol of pH around 7.2 [42], a predominant form of inorganic antimony would be $Sb(OH)_3$, which might be a poor substrate for Acr3p. Yet, we cannot exclude the possibility that weaker induction of Acr3p expression in the presence of Sb(III) is also responsible for a minor role of this permease in conferring Sb(III) resistance.

Expression of Acr3p in yeast is principally regulated at the transcriptional level in a (Figs. 2–4) [35–37] and As(III) resistance can be significantly increased by the presence of extra copies of the *ACR3* gene [25–27] or by the relocation of the *ACR3* gene from its native subtelomeric locus into non-silenced region of the yeast genome (Sloma E., Wysocki R., unpublished results). As shown in Figs. 2 and 3, 0.1 mM As(III) robustly induced expression of Acr3p-GFP from the plasmids, while addition of 1 mM Sb(III) resulted in much lower levels of *ACR3-GFP* mRNA and its protein. This implies that Sb(III) is much weaker inducer of the *ACR3* promoter than As(III). However, expression of Acr3p-GFP from both low-copy and multi-copy plasmids fully complemented high Sb(III) sensitivity in the *ycf1*Δ mutants (Fig. 1, Table 2) and resulted in reduced Sb(III) uptake and high rate of Sb(III) efflux (Fig. 5). This data suggests that higher levels of Acr3p are fully capable of Sb(III) removal from yeast cells. On the other hand, our preliminary *in vitro* experiments on Acr3p-mediated metalloid transport across plasma membranes indicate that Acr3p has much greater capacity for As(III) than for Sb(III) efflux (Maciaszczyk-Dziubinska E., Migocka M., Wysocki R., unpublished results). Nevertheless, more studies are needed to elucidate both mechanism of metalloid transport conducted by Acr3p and what is the true form of its metalloid cargo. It would be also interesting to see whether other members of Acr3 family overexpressed in yeast will complement Sb(III) sensitivity and restore Sb(III) efflux in mutants lacking Ycf1p and Acr3p. This would provide crucial information whether ability to transport both As(III) and Sb(III) is conserved in the Acr3 family from bacteria to animals. However, considering the fact that Sb(III) is present in the environment at trace or ultratrace levels [44], one may hypothesize that the family of Acr3 proteins has been primarly developed as a mean to cope with As(III) toxicity.

Acknowledgements

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